Postprint: Research on Apoplast Antifreeze Proteins in Cabbage-Type Winter Rapeseed

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Abstract

To elucidate the cold resistance mechanism of winter rapeseed, field and pot experiments were conducted to detect the antifreeze activity of apoplastic proteins from leaves and roots of the low-temperature-stressed Brassica-type winter rapeseed variety 'Longyou 6'. Apoplastic proteins were separated by SDS-PAGE, and some highly expressed proteins were identified using MALDI-TOF/TOF mass spectrometry. The results showed that after cold acclimation in an artificial climate chamber, the apoplastic protein content in leaves of 'Longyou 6' increased significantly ($P<0.05$), reaching 92.31 $g \cdot g^{-1}$(FW) on day 5, which was 246.12% higher than the control. Although the apoplastic protein content decreased on days 10 and 15 of cold acclimation compared to day 5, it remained significantly higher than the control ($P<0.05$). On days 20 and 25 of cold acclimation, the apoplastic protein content continued to increase significantly, reaching its maximum value on day 25 ($P<0.05$). After 10 days of recovery growth at room temperature, the apoplastic protein content decreased significantly compared to before ($P<0.05$), but remained higher than both the control and day 10 of cold acclimation. During the cold acclimation process, obvious protein accumulation occurred in the leaf apoplast of 'Longyou 6'; after 10 days of recovery growth at room temperature, the protein content in the apoplast decreased significantly, indicating that the apoplastic proteins of cold-acclimated 'Longyou 6' are low-temperature-induced proteins. Antifreeze activity assays revealed that they possess recrystallization inhibition activity. Mass spectrometry identification revealed multiple proteins with unclear functions, among which β-1-3-glucanase was consistent with the antifreeze protein reported in winter rye. Recovery and antifreeze activity testing of the glucanase-like protein detected by mass spectrometry revealed it has weak ice crystal morphology modification effects, indicating that this glucanase-like protein is a low-activity antifreeze protein. Under low temperature stress, antifreeze proteins were synthesized and secreted into the apoplast of winter rapeseed leaves and roots, playing a positive role in resisting external low temperatures. Simultaneously, it is specu-

lated that there may be multiple undiscovered antifreeze proteins in the apoplast of super-cold-resistant winter rapeseed.

Full Text

Study on Apoplast Anti-Freeze Proteins in Winter Turnip Rape (Brassica rapa L.)

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Abstract

To elucidate the cold resistance mechanism of winter rapeseed, field and pot experiments were conducted to examine the antifreeze activities of apoplast proteins in the leaves and roots of the winter turnip rape cultivar 'Longyou 6' under low temperature stress. Apoplast proteins were separated by SDS-PAGE, and some highly expressed proteins were identified using MALDI-TOF/TOF mass spectrometry. The results showed that after cold acclimation in an artificial climate chamber, the apoplast protein content in 'Longyou 6' leaves increased significantly ($P<0.05$), reaching 92.31 $\mu g \cdot g^{-1}$ (FW) on day 5, which was 246.12% higher than the control. Although the apoplast protein content decreased on days 10 and 15 compared with day 5, it remained significantly higher than the control ($P<0.05$). The apoplast protein content continued to increase significantly on days 20 and 25 of cold acclimation ($P<0.05$), reaching its maximum value on day 25 ($P<0.05$). After 10 days of recovery growth at room temperature, the apoplast protein content decreased significantly ($P<0.05$) but remained higher than both the control and day 10 of cold acclimation. During cold acclimation, obvious protein accumulation occurred in the apoplast of 'Longyou 6' leaves, while protein content decreased significantly after 10 days of recovery at room temperature, indicating that these apoplast proteins in cold-acclimated 'Longyou 6' are low temperature-induced proteins. Antifreeze activity assays revealed recrystallization inhibition activity. Mass spectrometry identified various proteins with unclear functions, among which β-1,3-glucanase was consistent with antifreeze proteins reported in winter rye. Recrystallization inhibition tests on the recovered glucanase-like protein showed weak ice crystal morphology modification, suggesting that this glucanase-like protein is a low-activity antifreeze protein. Winter rapeseed synthesizes and secretes antifreeze proteins in the apoplast of leaves and roots under low temperature stress, which play a positive role in resisting external low temperatures. It is also speculated

that multiple undiscovered antifreeze proteins may exist in the apoplast of super cold-resistant winter rapeseed.

Keywords: Winter turnip rape; Apoplast; Antifreeze protein; Glucanase; SDS-PAGE; Mass spectrometry

Introduction

Winter rapeseed is an important winter oil crop in China, but severe cold climate seriously restricts its development in northern China. Since Sun et al. [1] proposed the feasibility of moving winter rapeseed northward to arid and cold regions of northern China, the planting area of winter rapeseed has been expanding, generating significant ecological and economic benefits. The successful northward expansion of winter rapeseed mainly depends on the successful breeding of super cold-resistant varieties. The Longyou series of Brassica rapa winter rapeseed varieties exhibit excellent cold resistance, but their cold resistance mechanism remains unclear. Research on antifreeze proteins (AFPs) in winter rapeseed provides a theoretical basis for further elucidating the cold resistance mechanism and northward expansion of winter rapeseed.

In 1992, Griffith et al. [2] first demonstrated the presence of antifreeze proteins in the apoplast of cold-acclimated winter rye (Secale cereale), pioneering research on antifreeze proteins in plants. Hon et al. [3] performed SDS-PAGE electrophoresis on winter rye apoplast extracts and directly recovered protein bands from the gel for study, discovering at least four antifreeze polypeptides. Although antifreeze protein activity can be lost during 2-ME reduction and boiling pretreatment of SDS-PAGE samples, Hon et al. found that antifreeze polypeptides recovered from SDS-PAGE gels retained ice crystal morphology modification function after acetone precipitation, a method that has become the simplest approach for isolating antifreeze proteins. Studies have shown that many overwintering plants contain antifreeze proteins with characteristics similar to fish AFPs, playing important roles in enhancing plant cell freezing resistance. Subsequently, researchers discovered AFPs in various plants including Ammopiptanthus mongolicus [4-5], the alpine plant Rhodiola algida var. tangutica [6], carrot (Daucus carota) [7], Polygonum viviparum [8], and winter wheat (Triticum aestivum) [9].

Our previous studies indicated that cold-acclimated winter rapeseed can maintain root cells in solution state (supercooled state) below freezing point, suggesting that antifreeze proteins may exist in winter rapeseed [10]. Recrystallization inhibition (RI) activity is an important characteristic of antifreeze proteins. Recrystallization refers to the redistribution of already formed ice crystal particle sizes, where some ice crystals grow larger while others become smaller, with large crystals continuing to grow and small ones eventually disappearing. As a class of proteins that can directly interact with ice crystals, antifreeze proteins (AFPs) have functions such as modifying ice crystal morphology and inhibiting ice recrystallization [11], playing positive roles in helping animals and plants

resist external low temperature damage. However, few studies have reported on the presence of antifreeze proteins in winter rapeseed.

This study employed microscopic observation of ice crystal recrystallization effects to test the antifreeze activity of apoplast crude extracts from winter rapeseed leaves and roots after cold acclimation, combined with SDS-PAGE and protein tandem mass spectrometry identification techniques to investigate the antifreeze activity of apoplast antifreeze proteins in winter rapeseed, aiming to provide a scientific basis for studying the cold resistance mechanism of Brassica rapa winter rapeseed.

Materials and Methods

1.1 Experimental Materials

This study used the super cold-resistant winter rapeseed cultivar 'Longyou 6' as experimental material, with seeds provided by the College of Agronomy, Gansu Agricultural University.

1.2.1 Treatment of Experimental Materials

Field planting: At the Shajingyi experimental base in Lanzhou, Gansu Province, seeds were sown on August 15, 2013, with conventional field management. Fresh leaves were collected on October 5 (temperature 19°C), November 15 (temperature 12°C), and December 1 (temperature 5°C to -6°C) for protein extraction.

Pot experiment: Using ordinary 15 cm × 13 cm nutrient pots with nutrient soil as substrate, seeds were sown at room temperature. When seedlings reached the 5-6 leaf stage, experimental treatments began. **Phase 1:** Room temperature non-acclimation treatment (NA) with 14 h light at 25°C and 10 h darkness at 20°C for 7 days before sampling. **Phase 2:** Cold acclimation treatment (CA) with 8 h light at 6°C and 16 h darkness at 4°C for 25 consecutive days, with sampling every 5 days. **Phase 3:** Room temperature recovery growth (deacclimation, NA) with 14 h light at 25°C and 10 h darkness at 20°C for 10 days before sampling. After sampling, proteins were extracted with three replicates.

1.2.2 Extraction and Quantification of Apoplast Proteins

Based on the method of Gong et al. [12] with slight modifications: Leaves were first rinsed with running water, washed twice with ddH_2O, surface moisture was absorbed with filter paper, and leaves were cut into strips approximately 1.0 cm long. Exudates were removed by washing with extraction buffer (50 mmol · L^{-1} Tris-HCl pH 8.0, 10 mmol · L^{-1} EDTA, 20 mmol · L^{-1} Vc). Leaves were immersed in this buffer and vacuum infiltrated at room temperature for 30 min, then surface solution was removed with filter paper. Leaves were placed in a 50 mL syringe, which was placed in a centrifuge tube and balanced before

centrifugation at 7,000 $r \cdot min^{-1}$ for 20 min. The solution collected at the bottom of the centrifuge tube was the apoplast crude extract.

Protein content was determined using the Coomassie brilliant blue method [13].

1.2.3 SDS Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Guo [14] using 4% stacking gel and 15% separating gel.

1.2.4 Determination of Antifreeze Activity in Apoplast Crude Extracts

The microscopic observation method of ice crystal recrystallization effects was used to test the antifreeze activity of apoplast crude extracts from winter rapeseed leaves and roots after cold acclimation, based on the method of Knight et al. [15] with slight modifications.

1.2.5 Recovery of SDS-PAGE Protein Bands

The method of Hon et al. [3] was followed. After SDS-PAGE electrophoresis, gels were washed with cold ddH_2O, stained with 0.1% (w/v) Coomassie brilliant blue R-250, and destained. Corresponding gel bands were excised with a sharp blade, crushed, placed in dialysis bags, and eluted with 5 volumes of elution buffer containing 25 $mmol \cdot L^{-1}$ Tris pH 8.3, 192 $mmol \cdot L^{-1}$ Gly, and 0.1% SDS at a constant current of 100 mA until the gel became colorless. The eluate was centrifuged at 12,000 $r \cdot min^{-1}$ for 30 min, the supernatant was ultrafiltered and lyophilized. The precipitate was diluted to an appropriate concentration for antifreeze activity determination.

1.2.6 Differential Protein Mass Spectrometry Analysis

Differential bands were excised from the electrophoretogram, destained, and digested before analysis using an ABI tandem time-of-flight mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer). Mass spectrometry data were searched against the NCBInr (NCBI-National Center for Biotechnology Information non-redundant database) using the Mascot program (http://www.matrixscience.com). Protein sequences meeting mass spectrometry identification requirements were functionally classified in the Gene Ontology database (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi).

Results

2.1 Apoplast Protein Content in 'Longyou 6' Leaves During Cold Acclimation

As shown in [Figure 1: see original paper], the apoplast protein content in 'Longyou 6'leaves grown at room temperature was 26.67 $\mu g \cdot g^{-1}$ (FW). After cold

acclimation in an artificial climate chamber, apoplast protein content increased significantly ($P<0.05$), reaching 92.31 $\mu g \cdot g^{-1}$ (FW) on day 5, representing a 246.12% increase over the control. Although apoplast protein content decreased on days 10 and 15 compared with day 5, it remained significantly higher than the control ($P<0.05$). Apoplast protein content continued to increase significantly on days 20 and 25 of cold acclimation ($P<0.05$). After seedlings were transferred back to room temperature for recovery growth (de-acclimation) for 10 days, apoplast protein content decreased significantly ($P<0.05$). During cold acclimation, obvious protein accumulation occurred in the apoplast of 'Longyou 6' leaves, while protein content decreased significantly after de-acclimation, indicating that the accumulated proteins in leaf apoplast under low temperature are closely related to cold acclimation treatment in rapeseed.

2.2 SDS-PAGE Analysis of Apoplast Proteins in 'Longyou 6' During Cold Acclimation

Based on the analysis of apoplast protein content in 'Longyou 6' leaves during cold acclimation, further SDS-PAGE analysis was conducted. As shown in [Figure 2: see original paper]a, electrophoretic analysis was consistent with protein quantification results. At room temperature, apoplast protein content in leaves was low (lane NA in [Figure 2: see original paper]a). After cold acclimation, both the types and expression levels of proteins increased significantly, particularly a protein with molecular weight around 38 kD (S1), whose expression decreased significantly after de-acclimation, indicating that this protein is closely related to cold stress and could be a candidate antifreeze protein.

To verify the reliability of pot experiment results, apoplast proteins from leaves and roots of field-grown 'Longyou 6' were analyzed by electrophoresis. SDS-PAGE analysis showed ([Figure 2: see original paper]b) consistent results with those obtained from artificial climate chamber cold acclimation treatment. Due to the limited amount of apoplast protein obtained from artificial climate chamber treatment, field-grown 'Longyou 6' during winter was mainly used for subsequent studies.

2.3 Antifreeze Activity Analysis of Apoplast Proteins in Cold-Acclimated 'Longyou 6'

The microscopic observation method of ice crystal recrystallization effects was used to analyze ice crystal growth characteristics in apoplast crude extracts from 'Longyou 6' leaves and roots. As shown in [Figure 3: see original paper], compared with ice crystal growth in apoplast crude extracts from non-acclimated 'Longyou 6' (Figures 3A and 3C), ice crystals in apoplast crude extracts after cold acclimation were significantly smaller, more uniform, and more numerous (Figure 3B), indicating that leaf apoplast protein crude extracts have recrystallization inhibition activity. Figure 3D shows ice crystal growth in apoplast crude extracts from cold-acclimated 'Longyou 6' roots, demonstrating extremely strong recrystallization inhibition activity.

2.4 Sampling and Mass Spectrometry Identification of Apoplast Proteins in Cold-Acclimated 'Longyou 6'

Based on the SDS-PAGE electrophoretogram of apoplast proteins from leaves and roots of field-grown winter rapeseed 'Longyou 6' (samples from December 1), highly expressed polypeptides were excised for tandem mass spectrometry (MALDI-TOF-TOF-MS) identification. Sampling numbers are shown in [Figure 4: see original paper]. Fifteen differential proteins were identified through mass spectrometry and Mascot searching, with results presented in . These proteins could be classified into two functional categories based on their biological functions: disease defense proteins (6 proteins) and metabolism-related proteins (9 proteins). Among them, subtilisin-like protease (Prl), myrosinase, heat shock proteins (HSPs), pathogenesis-related proteins (PRs), β-1,3-glucanase, and glucanase-like proteins belong to disease defense proteins, while phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, enolase, alcohol dehydrogenase, isocitrate dehydrogenase, S-adenosyl-L-homocysteine hydrolase, peroxidase, and glutathione transferase belong to metabolism-related proteins.

2.5 Recovery and Activity of Glucanase-Like Protein in Apoplast of 'Longyou 6'

SDS-PAGE results showed that the expression of glucanase-like protein (No. 11) in leaf apoplast during low temperature induction was well correlated with temperature changes. As a highly expressed protein in the apoplast with antifreeze protein-like behavior, and since β-1,3-glucanase has been proven to have antifreeze activity in winter rye and is an antifreeze protein [26], this protein was primarily selected for recovery and antifreeze activity testing. In fact, the SDS-PAGE gel of leaf apoplast crude extracts contained two glucanases with very similar molecular weights (Nos. 11 and 12 in [Figure 2: see original paper]b and [Figure 4: see original paper]), while roots contained only one of them (No. 11 in [Figure 4: see original paper]). These were two highly expressed proteins in low temperature-induced leaf apoplast proteins. Due to the close proximity of these two bands on the electrophoresis gel, they were not completely separated during recovery. Purity testing of the recovered product showed ([Figure 5: see original paper]) a relatively broad band on the SDS-PAGE gel, indicating that it may contain both glucanase-like proteins. Antifreeze activity testing of the recovered product showed that the glucanase-like protein had ice crystal morphology modification effects, with ice crystals growing in hexagonal shapes ([Figure 6: see original paper]), indicating it is a low-activity antifreeze protein. However, it remains unclear which of the two glucanase-like proteins has antifreeze activity or whether both possess antifreeze activity.

Discussion

3.1 Winter Rapeseed Synthesizes and Secretes Antifreeze Proteins Under Low Temperature Stress

This study preliminarily confirmed that protein accumulation occurs in the apoplast of winter rapeseed leaves and roots during cold acclimation. Antifreeze activity assays of apoplast crude extracts from cold-acclimated 'Longyou 6' leaves and roots showed obvious ice crystal morphology modification and recrystallization inhibition effects, indicating the presence of antifreeze proteins in the apoplast crude extracts of cold-acclimated winter rapeseed leaves and roots. During winter, after low temperature and short-day acclimation, some mechanism to avoid cell freezing is activated in strongly cold-resistant winter rapeseed, enabling root cells to maintain a non-frozen state under subfreezing temperatures, thereby avoiding lethal damage from intracellular ice formation and facilitating safe overwintering. Antifreeze proteins may play important roles in this process. Because antifreeze proteins are produced in the apoplast of winter rapeseed, they can modify ice crystal morphology and inhibit ice recrystallization, confining ice crystal formation to the apoplast, regulating cell supercooling state, and simultaneously avoiding mechanical damage to cells from large ice crystals.

3.2 Discussion on Functions of Apoplast Proteins in Cold-Acclimated 'Longyou 6'

Proteins detected by mass spectrometry could be classified into two functional categories: disease defense proteins (6 proteins) and metabolism-related proteins (9 proteins).

3.2.1 Disease Defense Proteins Subtilisin-like protease (Prl) can degrade insect cuticle and is a virulence factor produced by entomopathogenic fungi when infecting hosts [16]. Myrosinase primarily functions as an important component of the glucosinolate-myrosinase system, with its most important function being mediating interactions between plants and other biological environments, thereby affecting plant self-defense [17-18]. Heat shock proteins (HSPs) are induced by various stresses (high/low temperature, drought, etc.) and have antioxidant and "molecular chaperone" functions within cells [19]. Pathogenesis-related proteins (PRs) are a class of proteins in plants induced by pathogens or many other external factors, playing important roles in plant responses to external pressures and disease resistance, with glucanase (PR-2) being one of them. The expression of β-1,3-glucanase is not only related to pathogen infection but also associated with various other abiotic induction factors. PR-1 is also an important family of PR proteins, but its function in plants remains unclear [20-21].

3.2.2 Metabolism-Related Proteins Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and enolase are important enzymes in the glycolysis

pathway, but their functions under low temperature stress have not been reported. Alcohol dehydrogenase is one of the important hydrolases in plant non-normal respiratory chains, having significant regulatory effects on alcohol metabolism and being related to reactive oxygen species scavenging and lipid peroxidation protection in plants [21]. Isocitrate dehydrogenase is a regulatory enzyme in the tricarboxylic acid cycle pathway, but recent studies have found that it participates in response processes to various stress conditions and plays an important role in plant antioxidant stress [22]. S-adenosyl-L-homocysteine hydrolase (SAHH) primarily participates in methylation processes in cellular metabolism and plays an important regulatory role in cellular lipid metabolism. Some studies have shown that SAHH may improve low temperature stress tolerance by regulating membrane lipid composition under low temperature [23]. Peroxidase (POD) is one of the key enzymes in the plant antioxidant enzyme system under stress conditions [24]. Glutathione transferase (GST) functions in plant primary metabolism, secondary metabolism, and cell signal transduction processes, thereby affecting plant growth and development, and is induced by stress [25].

3.3 Glucanase-Like Protein in Apoplast of Cold-Acclimated 'Longyou 6' May Be an Antifreeze Protein

Through direct separation of apoplast antifreeze proteins by denaturing SDS-PAGE gel electrophoresis, combined with MALDI-TOF/TOF mass spectrometry identification and microscopic observation of recrystallization effects, the recovered glucanase-like protein from SDS-PAGE gel showed certain recrystallization inhibition activity, indicating that the glucanase-like protein in the apoplast of cold-acclimated 'Longyou 6' leaves may be a weakly active antifreeze protein, similar to antifreeze polypeptides discovered in winter rye. Although it remains unclear which of the two glucanase-like proteins has antifreeze activity or whether both possess antifreeze activity, we believe No. 11 should be an antifreeze protein because its expression increased significantly during cold acclimation and decreased significantly after de-acclimation. Its function requires further verification.

3.4 Multiple Unknown Antifreeze Proteins May Exist in Strongly Cold-Resistant Winter Rapeseed

Multiple antifreeze proteins with various activities have been discovered in the apoplast of winter rye, and more than one antifreeze protein has also been found in the strongly freeze-tolerant plant Ammopiptanthus mongolicus [5,26]. In this study, SDS-PAGE gel electrophoresis showed that relatively abundant protein types exist in the apoplast of winter rapeseed, particularly in roots, and the apoplast crude extracts from roots showed extremely strong recrystallization inhibition activity. Therefore, it is speculated that multiple different antifreeze proteins may also exist in strongly cold-resistant winter rapeseed. This requires more in-depth experimental verification, including separation and purification

of apoplast proteins from roots and individual study of each protein. Analysis of mass spectrometry identification results of low temperature-induced apoplast proteins in winter rapeseed showed that the localization of some proteins was not clear. Whether certain low-expression proteins resulted from intracellular protein leakage due to cell membrane damage caused by low temperature or other factors requires further verification. No reports exist in available literature on these proteins being present in apoplast space, such as isocitrate dehydrogenase, alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. Based on existing information, although many proteins are related to cold stress, it is impossible to determine which proteins are antifreeze proteins. Some proteins have clear localization in cells, existing only in the apoplast, and their expression levels increase during low temperature induction, so these proteins must be related to cold stress, such as pathogenesis-related proteins like glucanase. These cold-induced apoplast proteins are distributed in both leaves and roots, but with different expression levels. Additionally, many studies have shown that antifreeze proteins in the apoplast are often bifunctional proteins [26]. Whether this is also the case for antifreeze proteins in winter rapeseed requires further research.

This study found that during cold acclimation, obvious protein accumulation occurred in the apoplast of 'Longyou 6' leaves, while protein content decreased significantly after 10 days of recovery growth at room temperature, indicating that apoplast proteins in cold-acclimated 'Longyou 6' are low temperature-induced proteins. The antifreeze activity of apoplast crude extracts from Brassica rapa winter rapeseed leaves and roots was detected using microscopic observation of ice crystal recrystallization. Apoplast proteins were separated by SDS-PAGE and some highly expressed proteins were identified using MALDI-TOF/TOF mass spectrometry. The study found that apoplast crude extracts from cold-acclimated Brassica rapa winter rapeseed showed obvious recrystallization inhibition activity, proving the existence of antifreeze proteins in the apoplast of cold-acclimated winter rapeseed leaves and roots. Among them, glucanase-like protein was consistent with antifreeze proteins reported in winter rye. Recovery and antifreeze activity testing of this glucanase-like protein showed it has weak recrystallization inhibition activity, indicating it is a low-activity antifreeze protein. It is speculated that multiple undiscovered antifreeze proteins may exist in the apoplast of strongly cold-resistant Brassica rapa winter rapeseed during winter.

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